Neuron
Article

Cell-Nonautonomous Function of Ceramidase in Photoreceptor Homeostasis

Jairaj K. Acharya,[1] Ujjaini Dasgupta,[2] Satinder S. Rawat,[2] Changqing Yuan,[1] Parthena D. Sanxaridis,[3] Ikuko Yonamine,[2] Pusha Karim,[2] Kunio Nagashima,[4] Michael H. Brodsky,[2] Susan Tsunoda,[3] and Usha Acharya[2,*]
[1]Laboratory of Cell and Developmental Signaling, National Cancer Institute, Frederick, MD 21702, USA
[2]Program in Gene Function and Expression, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
[3]Department of Biology, Boston University, Boston, MA 02215, USA
[4]EM Facility/Image Analysis Laboratory, SAIC Frederick, MD 21702, USA
*Correspondence: usha.acharya@umassmed.edu
DOI 10.1016/j.neuron.2007.10.041

SUMMARY

Neutral ceramidase, a key enzyme of sphingolipid metabolism, hydrolyzes ceramide to sphingosine. These sphingolipids are critical structural components of cell membranes and act as second messengers in diverse signal transduction cascades. Here, we have isolated and characterized functional null mutants of *Drosophila* ceramidase. We show that secreted ceramidase functions in a cell-nonautonomous manner to maintain photoreceptor homeostasis. In the absence of ceramidase, photoreceptors degenerate in a light-dependent manner, are defective in normal endocytic turnover of rhodopsin, and do not respond to light stimulus. Consistent with a cell-nonautonomous function, overexpression of ceramidase in tissues distant from photoreceptors suppresses photoreceptor degeneration in an arrestin mutant and facilitates membrane turnover in a rhodopsin null mutant. Furthermore, our results show that secreted ceramidase is internalized and localizes to endosomes. Our findings establish a role for a secreted sphingolipid enzyme in the regulation of photoreceptor structure and function.

INTRODUCTION

Sphingolipids are essential structural components of membranes and regulate membrane architecture (Holthuis et al., 2001). Many sphingolipids, such as ceramide, sphingosine, and sphingosine 1-phosphate, are also bioactive molecules that regulate diverse cellular processes, including growth, differentiation, apoptosis, and angiogenesis, among others (Dickson, 1998; Futerman and Hannun, 2004; Hla, 2004; Spiegel and Milstien, 2003). Generally, ceramide and sphingosine promote apoptosis and inhibit proliferation, while sphingosine 1-phosphate promotes growth and inhibits apoptosis (Hannun and Obeid, 2002; Le Stunff et al., 2004). Ceramide is a precursor for sphingosine, sphingosine 1-phosphate, sphingomyelin, and complex sphingolipids. Enzymes involved in the generation and conversion of these sphingolipids are conserved across species (Futerman and Riezman, 2005). Ceramidases (CDases) hydrolyze ceramide to sphingosine, which is subsequently phosphorylated to sphingosine 1-phosphate. CDases are thus key enzymes that attenuate ceramide-mediated effects and regulate ceramide/sphingosine/sphingosine 1-phosphate levels in cells. CDases are classified as acid, neutral, or alkaline based on their pH optimum. A deficiency of acid CDase causes Farber's disease in humans, in which ceramide accumulates in the lysosomes (Sugita et al., 1972). *Drosophila* has one neutral CDase and no known acid CDase homolog (Acharya and Acharya, 2005).

Drosophila phototransduction, a prototypic G protein-coupled receptor (GPCR) signaling cascade, is initiated when the visual pigment rhodopsin absorbs light (Hardie and Raghu, 2001; Montell, 1999; Zuker, 1996). Rhodopsin activates a heterotrimeric G protein, Gq, and the effector for Gq is phospholipase C (NorpA). Activation of NorpA leads to the opening of light-sensitive cation channels (Trp and Trpl), by a mechanism that is not completely understood. Termination of the cascade is achieved by the binding of arrestins (Arr1 and Arr2) to rhodopsin, leading to its inactivation.

In the current study, we show that CDase acts in a cell-nonautonomous manner to maintain photoreceptor homeostasis. CDase null mutations, obtained by chemical mutagenesis, are embryonic lethal. Clones of *cdase* null mutant photoreceptors generated in a CDase heterozygous background do not degenerate. However, we have identified a genetic background from which *cdase* null adult flies can be recovered. The photoreceptors of these CDase null flies undergo light-dependent degeneration, signifying that cell-nonautonomous function of CDase suppresses degeneration in mosaic mutant clones.

In earlier studies, we showed that targeted overexpression of ceramidase in photoreceptors suppresses retinal degeneration in certain *Drosophila* phototransduction mutants (*arrestin* and *norpA*) and facilitates the dissolution of incompletely formed rhabdomeric membranes in a rhodopsin mutant (Acharya et al., 2003, 2004). Here, we show that, consistent with a cell-nonautonomous function, targeted expression of CDase in a tissue distant from photoreceptors (such as the fat body or mushroom body) is capable of suppressing retinal degeneration in an *arrestin* mutant and accelerating the turnover of involuting rhabdomeric membranes in a *rhodopsin* mutant. We also show that secreted CDase is internalized from the cell surface and localizes

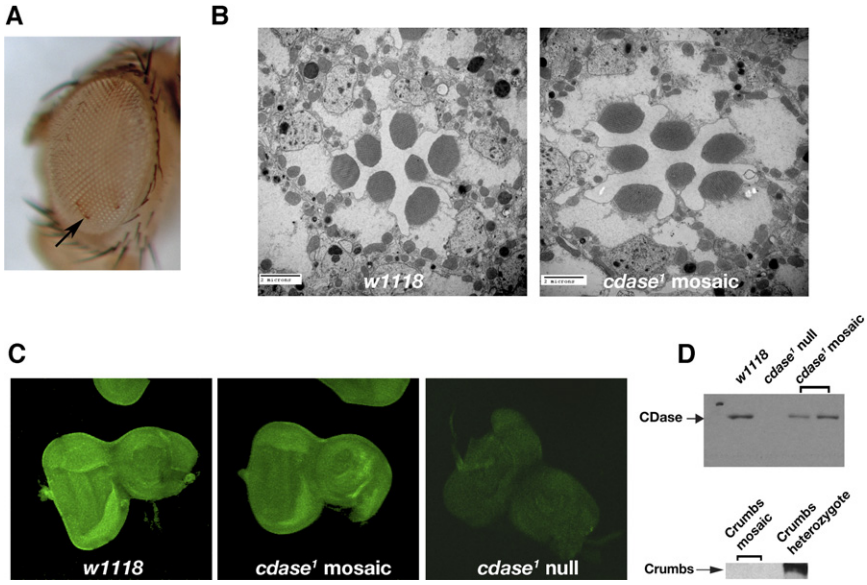

Figure 1. CDase Protein Is Still Present in *cdase*^{1} Mutant Clones

(A) *cdase*^{1} mosaic eye is generated using FRT-FLP recombination in conjunction with a cell lethal mutation on the wild-type chromosome. This resulted in an almost entire eye derived from mutant cells except for small red spots (marked by arrow) that are heterozygous for CDase.

(B) Transmission electron micrograph showing 15-day-old *w1118* and *cdase*^{1} clone of photoreceptors. Mutant photoreceptors generated by mitotic recombination in heterozygotes do not show signs of degeneration and are similar in structure and organization to wild-type photoreceptors.

(C) Eye imaginal discs from *w1118* and *cdase*^{1} mutant clones are stained with anti-CDase antibody. *cdase*^{1} mutant clones are immunopositive for CDase protein, while functional null *cdase*^{1} mutant (described later) does not show antibody reactivity.

(D) Western analysis of retinal extracts from *w1118*, *cdase*^{1}, and *crumbs* mutant clones reveals that CDase protein can be detected in *cdase* extract (upper panel), whereas *crumbs* extract generated similarly does not contain crumbs protein (lower panel). *cdase*^{1} null mutant described later does not have any CDase protein.

to the endosomes. Our data indicate that CDase participates in the normal endocytic turnover of the arrestin-rhodopsin complex in photoreceptors.

The findings that CDase acts in a cell-nonautonomous manner to promote the survival and function of photoreceptors may have important clinical applications for suppressing retinal degeneration in humans. In addition, these results provide direct evidence that sphingolipid metabolism plays important physiological roles both extracellularly, at the plasma membrane, and within the endocytic compartments.

RESULTS

CDase Null Mutations Are Embryonic Lethal

To isolate *cdase* mutants, we used a western blot-based ethylmethanesulfonate (EMS) mutagenesis screen described previously (Acharya et al., 2006). The screening strategy was based on the loss of CDase antigen in immunoblots from EMS-mutagenized flies that were viable over a deficiency that uncovers the CDase gene region (see Figure S1A available online). Lethal lines that were generated in the screen were carried over a balancer and subjected to transgenic rescue with a genomic copy of CDase. 2392 lines were established, of which 21 lines were lethal over the deficiency. Transgenic rescue experiments with these lines led to the isolation of the *cdase*^{1} mutation, which harbors a G to A transition at amino acid 641, converting a tryptophan residue into a stop codon (Figure S1B). The mutated gene encodes an extremely unstable protein, given that no endogenous protein corresponding to the mutant version is visible in western blots of *cdase*^{1} null flies (as described in section corresponding to Figure 2A). Because EMS causes random mutations throughout the chromosomes, all other incidental mutations within the *cdase*^{1} mutant were removed by three successive outcrossings to control *w1118* chromosomes.

The *cdase*^{1} mutation was a homozygous lethal; mutant animals died at late embryonic stages. Adult flies could be recovered when carrying a transgene with a genomic copy of CDase. To analyze the *cdase*^{1} lethal mutants, we generated mosaics in the eye using the ey-FLP/FRT system in combination with a cell-lethal mutation on the wild-type chromosome (Newsome et al., 2000). In these mosaics, almost the entire eye is homozygous for the mutation while the rest of the animal is heterozygous for CDase (Figure 1A).

cdase^1 Mutant Photoreceptors Generated by Mitotic Recombination Do Not Degenerate

Based on our earlier results showing that targeted overexpression of CDase rescues photoreceptor degeneration in certain phototransduction mutants, we predicted that CDase would play a significant role in photoreceptor homeostasis (Acharya et al., 2003). Therefore, we looked at *cdase*^{1} mosaic photoreceptors for ultrastructural abnormalities by transmission electron microscopy (TEM) (Figure 1B). Each of the 800 ommatidia of a *Drosophila* compound eye consists of eight photoreceptor cells (R1 to R8). Each cell has a rhabdomere, a specialized microvillar structure derived from the plasma membrane that houses the phototransduction machinery. Rhabdomere architecture is sensitive to perturbations in the phototransduction cascade and has been used to monitor photoreceptor degeneration (Montell, 1999). *cdase*^{1} mosaics aged to 15 days posteclosion showed a normal rhabdomere architecture (Figure 1B, right panel; compare with wild-type, left panel).

Because the *cdase*^{1} mosaic photoreceptors were morphologically normal, we performed immunocytochemistry on mosaic eye discs with antibodies to CDase to ascertain the lack of CDase protein in the developing eye (Figure 1C). Surprisingly, the *cdase*^{1} mosaic tissue still showed staining for CDase, despite being genetically null for this protein (Figure 1C, middle panel).

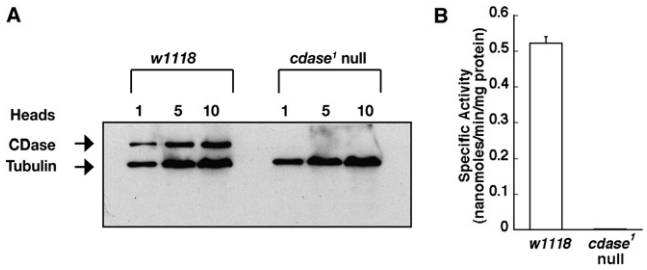

Figure 2. *cdase*^{1} Functional Null Mutant Is Devoid of CDase Protein and Activity

(A) Western blot analysis of one, five, and ten head extracts from *w1118* and *cdase*^{1} null reveals that no CDase protein is detected in mutant lanes. The blot is probed for tubulin as a loading control.

(B) Neutral CDase activity is carried out using [1-^{14}C] Palmitoyl sphingosine as the substrate and measuring radioactivity in the released fatty acid. While control *w1118* extract shows good CDase activity, *cdase*^{1} null extract does not show any activity. Each column represents the mean of four independent experiments, and the error bars denote the standard deviation.

Similarly, full-length CDase (not the truncated product encoded by the mutant message) was detected in western blots of retinal extracts from the mosaic eyes (Figure 1D). As a control for our method, we generated similar photoreceptor null mutations in a *crumbs* mutant, whose gene product, crumbs, is required for photoreceptor morphogenesis and maintenance (Izaddoost et al., 2002; Pellikka et al., 2002). Retinal extracts from *crumbs* mosaic tissue do not show any crumbs protein, unlike *cdase* mosaics that show CDase protein (Figure 1D).

We reasoned that our findings were due to CDase being a secreted protein (Tani et al., 2003; Yoshimura et al., 2002). Most likely, CDase synthesized elsewhere in the animal was transported to the mutant eye tissue. This interpretation could account for CDase staining observed in the homozygous null eyes and perhaps their lack of structural defects. In case of a nonsecreted protein like crumbs, genetically null clones are truly devoid of protein because crumbs will not be transported to the mutant cells from heterozygous tissues. The results of these mosaic analysis experiments also indicated that this approach was not a feasible way to analyze CDase function in vivo. Further study would require the development of a functional *cdase* null mutant that gave rise to viable adult flies.

Isolation of a Viable Functional Null Mutant of CDase

During the course of our studies, we observed that the original *cdase*^{1} chromosome, though a homozygous lethal, generated some viable progeny (less than 5%) when crossed to a deficiency that uncovers the CDase region, because of the presence of a closely linked suppressor mutation on the same chromosome. The frequency of viable flies was rescued to normal Mendelian ratios with the introduction of a genomic copy of CDase, which indicated that the CDase mutation contributed to the lethality in this genotype and that the suppressor mutation rescued some of the lethality. The identity of this viable suppressor mutation is currently unknown.

Further examination of the viable adults by western analysis of lysates with several polyclonal and monoclonal antibodies against CDase showed that these flies were devoid of CDase protein (Figure 2A). Furthermore, a measurement of neutral CDase activity showed no detectable activity (Figure 2B). Immunostaining of eye discs from mutant third-instar larvae of this line did not show any CDase immunoreactivity either (Figure 1B, last panel). Therefore, we concluded that these flies were functional null mutants for CDase. The identification of this viable CDase null mutant has permitted us to continue our in vivo studies.

cdase^{1} Functional Null Mutants Undergo Light-Induced Photoreceptor Degeneration

Given that photoreceptor-targeted overexpression of CDase rescues degenerating photoreceptors in certain phototransduction mutants (Acharya et al., 2003), we conducted photoreceptor structure-function studies with the *cdase*^{1} functional null mutants (hereafter called *cdase*^{1} null). The adult mutant flies were raised in regular light and dark cycles and maintained for varying numbers of days after eclosion; their photoreceptors were then examined by TEM. As shown in Figure 3A (second panel), 5-day-old flies showed severe photoreceptor degeneration. Many photoreceptor cells were missing their rhabdomeres and had vacuolated cell bodies. This degenerative phenotype was completely rescued by introducing a genomic copy of CDase, indicating that the loss of CDase was responsible for the observed photoreceptor degeneration (Figure 3A, third panel). While the suppressor mutation rescues some lethality, it is unable to compensate for lack of CDase in photoreceptors. One likely explanation is that the high turnover of membranes and signaling molecules in active photoreceptors may create a greater requirement of CDase in this cell type compared to others.

To find out whether the retinal degeneration of *cdase*^{1} null mutants depended on light activation of the phototransduction cascade, we raised mutant flies in constant darkness for varying periods and observed their photoreceptors by TEM. As expected for a light-dependent effect, photoreceptors of the dark-raised, 5-day-old mutant flies did not show morphological signs of degeneration (Figure 3A, last panel). This finding also ruled out the possibility that the photoreceptor degeneration was the result of a developmental defect. Furthermore, because CDase localizes to the rhabdomeres and the subrhabdomeric areas of wild-type photoreceptors (Figure 3B), it seems that CDase plays a local role in the survival and function of the photoreceptors.

CDase Functions in a Cell-Nonautonomous Manner to Suppress Photoreceptor Degeneration

The observation from mosaic experiments that CDase null photoreceptors in a heterozygous background did not degenerate (Figure 1B) had led us to believe that CDase, which is secreted, acts in a cell-nonautonomous manner (Figure 1C, middle and last panels).

To determine whether CDase expressed in a different tissue could indeed reach the photoreceptors, we expressed the enzyme using UAS-CDase transgenic lines in the fat bodies of wild-type flies using a fat-body-specific GAL4 line (Hrdlicka et al., 2002). (The fat body of *Drosophila* is the functional equivalent of mammalian liver and adipose tissue.) As expected, western analysis of extracts from dissected fat bodies of these flies show overexpression of CDase (Figure 4A, upper right panel). We also isolated retina from these fat-body-driven flies for

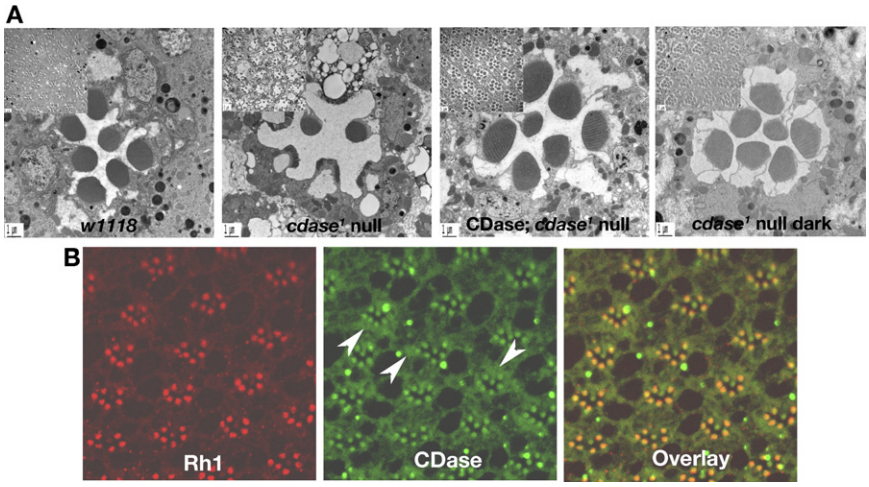

Figure 3. *cdase*^{1} Functional Null Mutant Photoreceptors Undergo Light-Dependent Degeneration

(A) TEM of 5-day-old photoreceptors of *w1118*, *cdase*^{1} null, *cdase*^{1} null rescue flies (with genomic CDase on second chromosome) reared in light, and *cdase*^{1} reared in dark. *cdase*^{1} null photoreceptors exposed to light show degeneration accompanied by loss of rhabdomeres and vacuolation of cells. Photoreceptor degeneration can be completely rescued in mutant flies expressing a genomic copy of CDase. *cdase*^{1} mutant flies raised in the dark do not show signs of degeneration seen in corresponding light-exposed flies. Insets show a low-magnification view of the photoreceptors of the corresponding genotype.

(B) CDase protein stains the rhabdomeres and subrhabdomeric region (marked by arrowheads) in wild-type photoreceptors. Thin sections of *w1118* photoreceptors are double stained for rhodopsin and CDase proteins with respective antibodies. In wild-type photoreceptors, CDase can be colocalized with rhodopsin as shown in the overlay.

western analysis. The blots showed that expression of CDase in the fat body resulted in significant accumulation of CDase in retinal lysastes (Figure 4A, lower right panel). In contrast, a nonsecreted protein, crumbs, when overexpressed under the control of the same fat body driver (Figure 4A, upper left panel), did not accumulate in the retina (Figure 4A, lower left panel).

We also visualized the fat-body-driven CDase that accumulates in the retinal tissue using the *cdase*^{1} null background. We isolated eye discs from larvae expressing fat-body-driven CDase in the *cdase*^{1} null and stained these discs for the protein. As shown in Figure 4B, mutant eye discs did not show CDase staining, while eye discs from mutants expressing CDase in the fat body displayed robust staining for CDase. Thus, protein made in the fat body reached the eye discs of the *cdase*^{1} null animals. To test whether CDase transported to photoreceptors is active, we measured enzyme activity after dissecting retina from *cdase*^{1} null flies and *cdase*^{1} null flies expressing fat-body-driven CDase. While the mutant shows no activity, retinal extracts from mutants expressing fat-body-driven CDase show high enzyme activity (Figure 4C). These results show that CDase that reaches the target site (i.e., photoreceptors) from a distant tissue is active in hydrolyzing ceramide.

We next examined whether CDase indeed functioned cell-nonautonomously, as we suspected. To do so, we asked whether overexpression of CDase by the fat body was sufficient to rescue the photoreceptor degeneration of an *arrestin* (*arr2*^{3}) mutant and promote membrane turnover in a *rhodopsin* null (*ninaE*^{I17}) mutant. As shown in Figure 4D, fat body expression of CDase in *arr2*^{3} suppressed photoreceptor degeneration (top panel), and its expression in *ninaE*^{I17} enhanced the turnover of involuting rhabdomeric elements (bottom panel). These results are similar to those obtained by expressing CDase specifically in the photoreceptors of these mutants (Acharya et al., 2003, 2004), and they demonstrate that extracellular CDase is indeed capable of modulating the phenotypes caused by these mutations. To further validate that CDase can function cell-nonautonomously, we performed similar experiments after ectopically overexpressing CDase in the mushroom body neurons (Schwaerzel et al., 2002). Mushroom bodies are centers of olfactory memory in *Drosophila*. As seen in Figure S3, here too, CDase reaches the retina (Figure S3A) and has similar effects in *arr2*^{3} (Figure S3B, upper panel) and *ninaE*^{I17} mutants (Figure S3B, lower panel).

Ceramide Levels Correlate with CDase Function in Photoreceptors

Our staining experiments indicate that extracellular CDase can be transported to the photoreceptors. Because CDase catalyzes the hydrolysis of ceramide to sphingosine and a fatty acid, we carried out experiments to test whether a decrease in substrate (ceramide) or formation of products (sphingosine or fatty acid) could be responsible for CDase function in this study. We used electrospray tandem mass spectrometry to estimate the ceramide levels in lipid extracts prepared from control, *cdase*^{1} null mutant, and fat-body-driven CDase in *cdase*^{1} null flies and identified molecular species of ceramide containing tetradecasphingenine by negative ion ESI/MS/MS, as described previously (Acharya et al., 2003; Thomas et al., 1999). Lipid extracts from the mutant flies showed an increase in total ceramide levels compared with those from wild-type controls, showing that one of the consequences of a CDase mutation is increased ceramide levels (Figure 5A). Overexpression of CDase in the fat body in CDase mutant background results in significant decrease in ceramide level (Figure 5A). CDase action not only decreases ceramide levels, it also leads to formation of sphingosine. To evaluate whether formation of sphingosine was responsible for CDase's action, we tested whether increased sphingosine could suppress the degeneration in *cdase*^{1} null mutant. Lace encodes for one of the subunits of *Drosophila* serine palmitoyltransferase, the first and rate-limiting enzyme of sphingolipid biosynthesis, and *lace* mutants are lethal (Adachi-Yamada et al., 1999). It has been shown earlier that viability of *lace* alleles deficient in de novo biosynthesis of sphingosine is promoted when they are raised in food

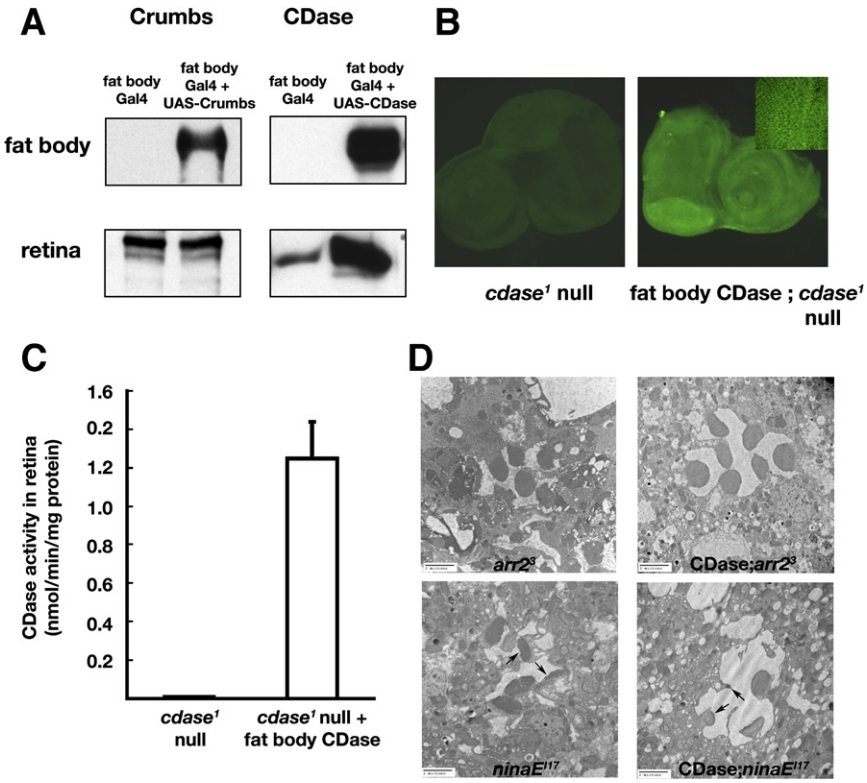

Figure 4. CDase Expressed in Fat Body Reaches Eye Discs, Is Active, Suppresses Degeneration in *arr2*^{3}, and Promotes Membrane Turnover in *ninaE*^{I17}

(A) CDase is overexpressed in *w1118* using a fat body Gal4 driver. Crumbs protein driven similarly in the fat body is used as control. The top panel shows overexpression of CDase (upper right panel) and crumbs (upper left panel) in extracts (10 μg protein) prepared from dissected fat bodies. Retina are also isolated from these flies, and extracts (10 μg protein) are subjected to western analysis. Retinal extracts show significant accumulation of CDase (lower right panel) but not crumbs (lower left panel).

(B) CDase is overexpressed using fat body Gal4 driver in *cdase*^{1} background. Eye discs are isolated from these flies (*cdase*^{1} null and *cdase*^{1} null expressing CDase) and stained with a monoclonal antibody to CDase. These discs show good staining for CDase compared to mutant discs that do not express fat-body-driven CDase. Inset shows that in addition to staining the cytoplasm, CDase staining is observed in punctate dots inside the cells.

(C) CDase expressed in the fat body is active in retina. Retinae are isolated from *cdase*^{1} null mutants and *cdase*^{1} null expressing fat-body-driven CDase. Neutral CDase activity is carried out using radiolabeled palmitoyl sphingosine as substrate and measuring the radioactivity in the released fatty acid. While *cdase*^{1} null retina show no activity, retina from *cdase*^{1} null expressing CDase in fat body show high activity. Each column represents the mean of four independent experiments, and the error bars denote the standard deviation.

(D) The top panel shows photoreceptors of *arr2*^{3} and *arr2*^{3} expressing fat-body-driven CDase. CDase expression suppresses degeneration seen in *arr2*^{3}. The bottom panel shows photoreceptors of *ninaE*^{I17} and *ninaE*^{I17} expressing fat-body-driven CDase. Expression of CDase facilitates turnover of involuting rhabdomeric membranes (marked by arrows) in the *ninaE*^{I17} null mutant.

supplemented with sphingosine. Using rescue of *lace* lethality as evidence for sphingosine function in vivo, we raised *cdase*^{1} null mutant flies in food supplemented with sphingosine and examined their photoreceptors by electron microscopy. Wild-type photoreceptors are not affected by feeding sphingosine (Figure 5B). Sphingosine feeding does not suppress degeneration observed in *cdase*^{1} null photoreceptors (compare Figures 5C and 5D). This experiment suggests that sphingosine on its own is not a likely candidate for the observed effects of CDase in the present study. N-linked saturated fatty acid is the second product of CDase action. While we cannot currently rule out the role for a fatty acid in this process, our unpublished observations with ceramide kinase mutant and its overexpression in CDase mutants support the notion that CDase's function correlates with ceramide levels rather than sphingosine or fatty acid (U.D. and U.A., unpublished data). Our observations that CDase reaches photoreceptors from other tissues combined with the fact that ceramides are very hydrophobic and found only in membranous structures lead us to propose that local action of CDase is responsible for its effect on photoreceptors.

Extracellular CDase Acts on Ceramide at the Cell Surface and Is Internalized

CDase might first function at the cell surface and then affect intracellular events, such as membrane turnover or endocytosis and/or function, after being internalized from the cell surface. To investigate CDase's mechanism of action, we tested whether extracellular CDase could bind ceramide on the cell surface in vitro. We stably expressed a V5 epitope-tagged CDase in Schneider cells. Protein expression in this cell line was induced, and the culture medium containing the tagged, extracellular CDase was collected and partially purified. The tagged CDase had ceramide-hydrolyzing activity, as demonstrated by the conversion of NBD-ceramide to NBD-fatty acid (Figure 6B, last panel).

To determine whether extracellular CDase in fact hydrolyzed ceramide at the cell surface, we knocked down endogenous CDase expression in S2 cells by RNAi (Figure 6A) and incubated the cells with fluorescent ceramide (C12 NBD-ceramide) to incorporate this analog into the plasma membrane (Marks et al., 2005). The RNAi-treated cells were pelleted, washed, and incubated with either V5-tagged CDase or buffer alone. The lipids were then extracted from the cells and separated by thin-layer chromatography. We found increased hydrolysis (about 40%) of the C12 NBD-ceramide in the cells that were incubated with the tagged CDase (NBD-Cer + CDase), compared with the control cells (NBD-Cer no CDase) (Figure 6B). Therefore, extracellularly added CDase metabolized the C12 NBD-ceramide at the cell surface.

In our fat body CDase expression experiment, we detected punctate intracellular staining of the CDase protein in eye discs,

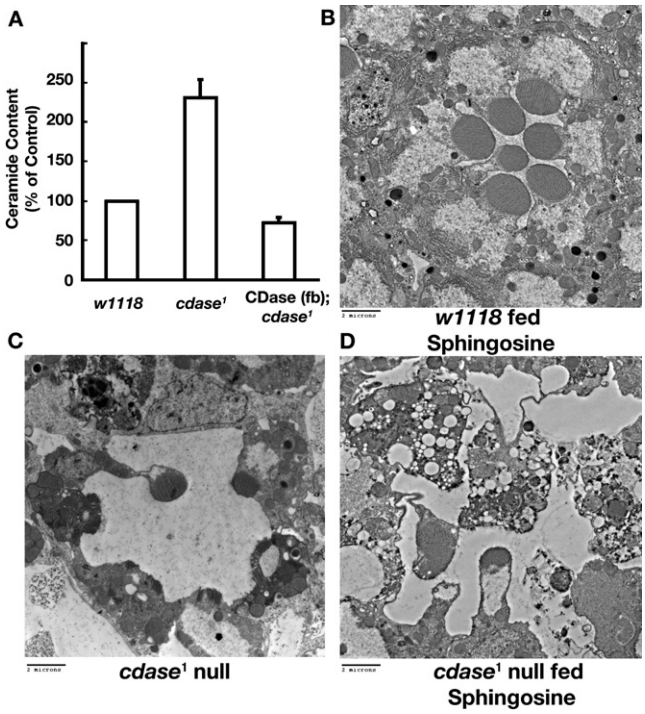

Figure 5. *cdase*[1] Null Shows Increased Ceramide Levels that Decrease Upon Overexpression of CDase, and Sphingosine Is Not Able to Rescue Photoreceptor Degeneration in *cdase*[1] Null Mutant

(A) Estimation of ceramide levels in control, *cdase*[1] null, and *cdase*[1] null expressing fat-body-driven CDase by mass spectrometry. Identification of ceramide molecular species containing tetradecasphingenine was performed by negative-ion ESI/MS/MS. Comparison of total ceramide shows that mutant flies have about 2-fold more ceramide than control. Expression of CDase in the fat body in the mutant background results in significant decrease in ceramide levels. Each column represents the mean of three independent experiments normalized to control *w1118*. The error bars in *cdase*[1] and CDase (fb); *cdase*[1] denote the standard deviation.

(B) Photoreceptors of *w1118* flies fed sphingosine. Control *w1118* flies were raised in food supplemented with N-acetyl-D-erythro sphingosine (14 carbon long chain base, native to *Drosophila*) at concentrations that rescued *lace* lethality. The eclosed flies were aged for 7 days, and their photoreceptors were observed by TEM. Rhabdomere architecture is not significantly altered in sphingosine fed wild-type flies.

(C) Photoreceptors of *cdase*[1] null aged for 7 days show extensive degeneration.

(D) Photoreceptors of *cdase*[1] null fed sphingosine. Sphingosine does not rescue photoreceptor degeneration in *cdase*[1] null flies.

which suggested that CDase might be internalized (see Figure 4B, inset). We also saw similar intracellular staining for CDase in normal eye imaginal discs (Figure S2). Therefore, we used S2 cells to investigate further whether extracellular CDase is internalized. We incubated S2 cells with the V5-tagged enzyme as the source of extracellular CDase and monitored the fate of the tagged enzyme by immunofluorescence analysis. The analysis showed the appearance of punctate fluorescent dots within the cytoplasm, which indicated that the extracellular CDase had been internalized (Figure 6C). To determine this, we stained S2 cells with markers of intracellular compartments and examined their localization relative to that of the V5-tagged CDase. The endocytic markers, Rab 11 (Figure 6D) dextran and *Drosophila* Rab5 (data not shown), colocalized with the internalized CDase, while a Golgi marker, lava lamp (Figure 6E), did not show colocalization (Dollar et al., 2002; Sisson et al., 2000; Wucherpfennig et al., 2003). Thus, extracellular CDase is internalized and sorted to the endosomes.

cdase[1] Mutants Transport Rhodopsin Normally to Rhabdomeres, but Show Defective Arr-Rh1 Interactions

Previous work on CDase has suggested that it functions in membrane-associated intracellular trafficking events. We showed that it facilitates membrane turnover in a rhodopsin null mutant (Acharya et al., 2004). Phenotypic studies on *cdase* deletion mutants generated by P element excision showed that CDase is important for synaptic function at larval neuromuscular junctions (Rohrbough et al., 2004). Because its mode of action is unknown, we set out to elucidate how CDase functions in photoreceptors.

During photoreceptor development, rhodopsin is transported to rhabdomeres through the secretory pathway. Several elegant studies have shown that proper transport of rhodopsin is crucial for photoreceptor stability (Colley et al., 1991; Kumar and Ready, 1995). As in vertebrates, *Drosophila* rhodopsin 1 (Rh1) is synthesized in the endoplasmic reticulum (ER) and glycosylated. It is then transported through different compartments of the Golgi complex where it undergoes further modification into mature Rh1 and is finally delivered to the rhabdomeres (Colley et al., 1991; Webel et al., 2000). The 34 kDa mature Rh1 is rhabdomeric; immature forms have a higher molecular weight (40 kDa) and are predominantly localized to the ER. Furthermore, several mutants where immature Rh1 accumulates, which is indicative of defective transport, show retinal degeneration (Colley et al., 1995; Kurada and O'Tousa, 1995).

To test whether the CDase mutants were defective in the forward transport of rhodopsin to the rhabdomeres, we looked for the accumulation of immature Rh1 in CDase mutants. As seen in Figure 7A, light-raised *cdase*[1] mutant flies showed reduced Rh1 levels by day 3 and its almost complete loss by day 10. This reduction is not surprising, as many degenerative mutations cause a precipitous drop in Rh1 levels. However, despite the reduction in Rh1 levels in *cdase*[1] mutants, only the mature, 34 kDa form of Rh1 was detected in the retinal lysates and not the immature 40 kDa form. We also looked at Rh1 levels in dark-raised flies, which do not show the morphological degeneration observed in light-raised flies. The Rh1 levels in the dark-raised *cdase*[1] mutants were similar to the levels in control flies, and the Rh1 detected was the mature form (Figure 7A). Thus, the *cdase*[1] mutants are not defective in the forward transport of Rh1 from the ER to rhabdomeres.

Invertebrate Rh1 is normally endocytosed upon light activation, and the two visual arrestins have been implicated in its endocytosis in *Drosophila* photoreceptors, a function similar to that of nonvisual mammalian arrestins, which are involved in the desensitization and endocytosis of activated GPCRs (Claing et al., 2002; Kiselev et al., 2000; Orem et al., 2006; Satoh and Ready, 2005; Stark et al., 1988). To test whether *cdase*[1] mutants are defective in Rh1 endocytosis, we used a biochemical assay that monitors the formation of a protein complex between Rh1 and

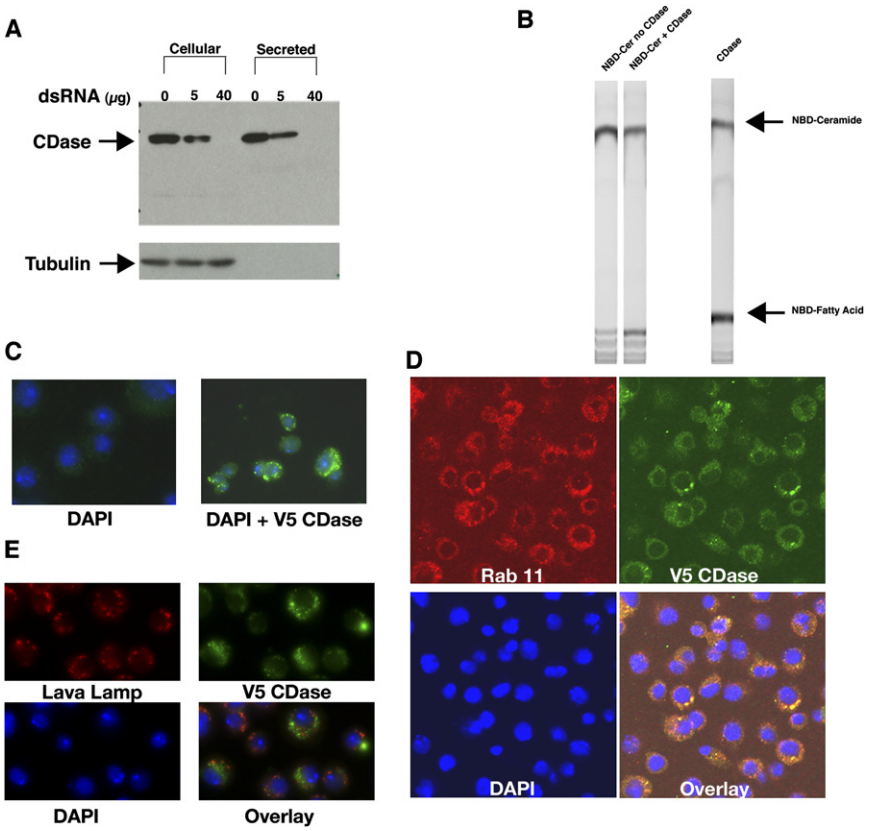

Figure 6. Extracellular CDase Can Bind Ceramide on Target Cells, Is Internalized; Internalized CDase Can Localize to the Endocytic Compartment

(A) Western analysis of RNAi-mediated knockdown of CDase in *Drosophila* S2 cells. The blot shows efficient reduction in both intra- and extracellular CDase upon treatment with dsRNA for 48 hr. CDase present in extracellular media is 7- to 8-fold more than that detected intracellularly. However, because the same amount of total protein was loaded in each lane and because extracellular media contains serum proteins, the blot does not reflect the actual distribution of CDase. Tubulin serves as a loading control for the blot.

(B) Cells treated with dsRNA as described in panel (A) are incubated with C12 NBD-ceramide. After incorporation of the fluorescent analog, cells are incubated with either V5-tagged partially purified CDase or buffer. After incubation, lipids are extracted and separated by TLC. 40% more NBD-ceramide is hydrolyzed to the product in cells that receive tagged CDase (NBD-Cer + CDase) compared to control cells (NBD-Cer no CDase). The rightmost TLC lane shows that tagged CDase used above is active in vitro in converting NBD-Cer to fatty acid.

(C) Immunofluorescence analysis of S2 cells incubated with V5-tagged CDase shows that it can be internalized. When stained with an antibody to V5, CDase can be visualized in punctate dots within the cells.

(D) Internalized CDase colocalizes with endocytic marker, Rab 11. S2 cells incubated with V5-tagged CDase are double stained with antibodies to *Drosophila* Rab11 and V5. DAPI is used to stain the nucleus. The overlay shows CDase localizes to structures that are positive for Rab11.

(E) Internalized CDase does not colocalize with lava lamp, a Golgi marker.

Arr2 in photoreceptors (Alloway et al., 2000). Blue light converts rhodopsin to active metarhodopsin, accompanied by the binding of arrestin 2. This binding is followed by endocytosis of the Rh1-Arr2 complex. Orange light converts Rh1 to an inactive form accompanied by the release of Arr2. We exposed wild-type and *cdase*^{1} dark-raised flies to blue or blue followed by orange light, prepared extracts, and fractionated them into supernatant and pellet fractions. In this assay, Arr2 bound to Rh1 pellets upon centrifugation, while released Arr2 is found in the supernatant. In the dark, Arr2 was found in the pellet and supernatant in both control and *cdase*^{1} null mutant photoreceptors (Figure 7B). When exposed to blue light, Arr2 bound Rh1 in both the control and the mutant flies (Figures 7B and 7C). However, upon photoconversion of Rh1 by orange light, Arr2 was efficiently released into the supernatant in the control whereas, in *cdase*^{1} null flies, Arr2 release was defective. The identical experiment performed with *cdase*^{1} mutants carrying a copy of genomic CDase (rescued flies) showed efficient release of Arr2 into the supernatant (as in Figures 7B and 7C).

We then tested the binding of Arr1 to Rh1 in the wild-type and mutant backgrounds using the same assay. We observed that in wild-type adult flies, unlike Arr2, Arr1 did not partition differently upon exposure to blue and orange light. Rather, almost all the Arr1 was found in the pellet fraction upon either blue or orange light exposure (Figure 7D). However, for the *cdase*^{1} mutants, more Arr1 was found in the supernatant than for wild-type flies (Figure 7D). This defect was rescued by introducing a genomic copy of CDase. Thus, in *cdase*^{1} null mutants, Arr1 associates less tightly with Rh1 than in wild-type flies. Thus, the dynamics of interaction between rhodopsin and arrestins are altered in *cdase*^{1} null mutants. These alterations could be indicative of defective endocytosis in *cdase*^{1} null photoreceptors.

cdase^{1} Mutant Photoreceptors Do Not Respond to Light

To test whether photoreceptor function in the *cdase*^{1} mutants was also defective, we performed electroretinogram recordings (ERGs) from control, mutant, and rescued flies. Because dark-raised *cdase*^{1} mutant flies did not show morphological degeneration, we performed the recordings on dark-raised flies. Interestingly, despite their intact photoreceptor morphology, the mutant flies did not respond to even high-intensity light stimulus (Figure 7D). However, the rescued *cdase*^{1} mutants showed normal ERGs. The block of phototransduction in the *cdase*^{1} mutants shows that CDase is critical for normal photoreceptor signal transduction. The lack of response to light in the absence of morphological changes suggests that CDase has additional roles in photoreceptor function beyond its effects on endocytosis.

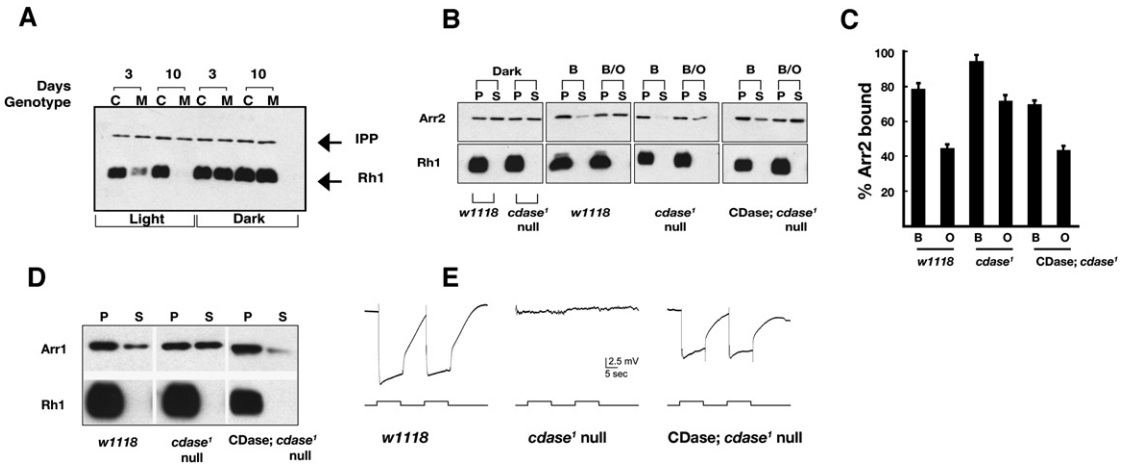

Figure 7. *cdase*[1] Null Is Not Defective in Forward Transport of Rh1 but Defective in Arr-Rh1 Interaction; *cdase*[1] Null Does Not Respond to Light

(A) Western analysis of Rh1 in light- and dark-raised *w1118* (C) and *cdase*[1] (M) retinal extracts. Rh1 level progressively decreases in light-exposed mutant photoreceptors, while dark-raised flies do not show decrease in Rh1 level. Rh1 is in the fully mature 34 kd form in the mutant photoreceptors. The blot is probed with an antibody to Inositol polyphosphate 1-phosphatase (IPP) as loading control.

(B) Biochemical analysis of endocytic turnover of Rh1-Arr complexes in photoreceptors. The panel shows Arr2 and Rh1 blots. In wild-type extracts, Arr2 can be pelleted with Rh1 in blue light and released efficiently to the supernatant in blue followed by orange light regimen. *cdase*[1] shows inefficient release of Arr2 under similar conditions, and this defect is rescued by the introduction of a wild-type copy of CDase. P and S represent pellet and supernatant fractions, respectively.

(C) Quantification of arrestin2 bound in control, *cdase*[1] null, and rescue flies. The graph shows that 27% more of the total Arr2 remains bound to Rh1 compared to control flies while it is comparable to wild-type in the rescued flies. Each column represents the mean of three independent experiments, and the error bars denote the standard deviation.

(D) In wild-type extracts, upon exposure to any light, most of Arr1 is in the pellet, while in *cdase*[1] null, a significant portion of it is in the supernatant. This defect is corrected in rescued flies.

(E) Electroretinogram (ERG) recordings from *w1118*, *cdase*[1] null, and Cdase; *cdase*[1] null flies. Dark-raised flies were exposed to two consecutive 10 s pulses of white light (400–700 nm, 6600 lux), as indicated beneath ERG recordings. Shown are representative ERG recordings from at least seven flies examined for each genotype. *cdase*[1] null flies displayed no light responsiveness at all light intensities (0.6–6600 lux) tested. As shown, CDase; *cdase*[1] null flies exhibited complete rescue of light responsiveness with signals indistinguishable from wild-type.

cdase[1] Mutants Show Excess Apoptosis in Eye Imaginal Discs

Kinetic evidence and the pharmacological manipulation of ceramide levels in mammalian cells have demonstrated a close link between ceramide levels and the induction of apoptosis (Hannun and Luberto, 2000; Hannun and Obeid, 2002). To detect apoptosis in the eye imaginal discs of third-instar *cdase*[1] larvae, we used an antibody against activated caspases. The anti-activated-caspase staining was stronger in the *cdase*[1] mutant imaginal discs than in wild-type discs (Figure 8A, top panel). This increased apoptosis was largely in the undifferentiated cells, anterior to the morphogenetic furrow. We then checked to see whether damage-induced apoptosis was enhanced in the *cdase*[1] mutants, as several studies in mammalian tissue culture have shown that stress stimuli, including ionizing radiation, lead to ceramide accumulation by interfering with enzymes involved in its metabolism (Hannun and Obeid, 2002; Kolesnick and Fuks, 2003). The imaginal discs of irradiated late third-instar larvae from *cdase*[1] mutants showed higher levels of caspase staining than those from irradiated wild-type controls (Figure 8A, bottom panel). Therefore, the *cdase*[1] null mutation results in an increased propensity for apoptosis compared with wild-type flies.

Because *cdase*[1] mutants are prone to apoptosis, we reasoned that the degeneration of mutant photoreceptors might be caused by apoptosis. To test this, we expressed the caspase inhibitor p35, using an eye-specific promoter, in *cdase*[1] mutants (Hay et al., 1994; Alloway et al., 2000). A TEM analysis showed that the expression of p35 in the mutant photoreceptors suppressed their degeneration (Figure 8B). Thus, activation of apoptosis mediates the degeneration observed in *cdase*[1] mutant photoreceptors.

DISCUSSION

In this study, we used a functional null mutant of *Drosophila* neutral CDase to show that this enzyme plays a critical role in regulating photoreceptor structure and function, cell-nonautonomously. Extracellular CDase is internalized from the cell surface, localizes to the endocytic compartment, and participates in the endocytic turnover of rhodopsin-arrestin complexes to maintain photoreceptor homeostasis.

In mutants such as *arrestin*, rhodopsin remains chronically active, leading to cytotoxicity and photoreceptor cell death. Retinal degeneration also occurs in a subset of visual system mutants (*norpA*, *rdgc*, *rdgb*) through the accumulation of an abnormally stable Rh1-Arr2 complex in the photoreceptor cells (Alloway et al., 2000; Kiselev et al., 2000). In this situation, the activated Rh1-Arr2 complexes undergo massive internalization and disrupt the endocytic pathway, but the exact mechanism by which

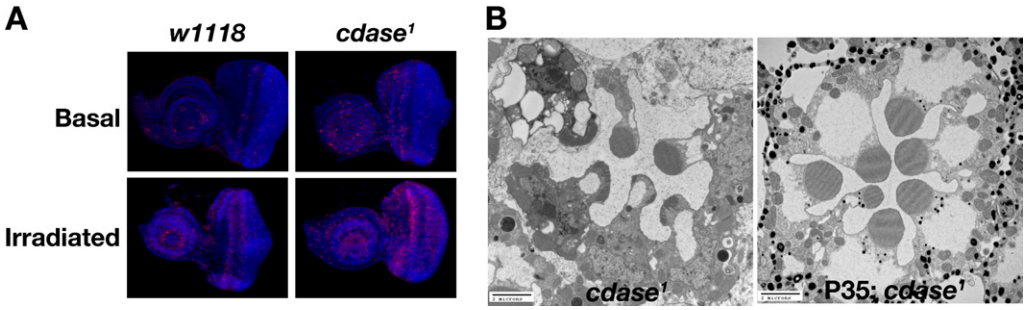

Figure 8. *cdase*^{1} Is Defective in Apoptosis, and Photoreceptor Degeneration in *cdase*^{1} Can Be Rescued by Expression of the Antiapoptotic Protein p35

(A) The top panel shows untreated eye discs from *w1118* and *cdase*^{1}, while the bottom panel shows eye discs that have been irradiated with X-rays, allowed to recover for 5 hr, and stained with anti-caspase antibody. Mutants reveal an increase in basal as well as damage-induced apoptosis as seen by the increased caspase staining in the discs.

(B) Expression of GMR-p35 in photoreceptors rescues degeneration in *cdase*^{1}. TEMs of 5-day-old photoreceptors are shown. Mutant photoreceptors show loss of rhabdomeres and vacuolation, these defects are not seen in mutant photoreceptors expressing p35.

these complexes impair endocytic function is not known. Nonetheless, the persistence of these complexes induces retinal degeneration, eventually leading to photoreceptor apoptosis. Although vertebrate rhodopsin is not normally endocytosed, recent studies show that certain mutations in rhodopsin associated with retinitis pigementosa (RP) cause the formation of stable rhodopsin-arrestin complexes that alter the morphology of the endosomal compartments and impair receptor-mediated endocytic functions (Chuang et al., 2004). Given CDase's ability to rescue similar mutations in flies, it may be possible to exploit this function clinically to suppress retinal degeneration in humans via the targeted overexpression of CDase.

Recent studies show that sphingolipid-metabolizing enzymes function both intracellularly and extracellularly. An isoform of sphingosine kinase 1 that acts on S1-P is secreted and contributes to establishing the vascular S1-P gradient that regulates angiogenesis (Ancellin et al., 2002). Similarly, our findings support both extracellular and intracellular roles for CDase. Furthermore, studies in mammalian tissue culture systems and in yeast have noted the modulation of endocytosis by ceramide or sphingoid base (Chen et al., 1995; D'Hondt et al., 2000). This is consistent with the idea that intracellular endocytic processes cannot proceed without cooperative changes in the outer leaflet of the plasma membrane bilayer; however, the nature of these changes has been largely unexplored. Our findings highlight the role of CDase in bringing about such cooperative changes. CDase-mediated alterations of the microenvironment of the lipid bilayer could potentially affect membrane dynamics, not only at the cell surface, where intracellular events would be initiated, but also in internalized compartments along the endocytic route. Recently, it has been demonstrated that ceramide intrinsically moves between the two leaflets of the plasma membrane efficiently (Mitsutake and Igarashi, 2007). Thus, changes in ceramide levels on the extracellular leaflet of membranes will affect ceramide levels in the cytoplasmic leaflet. This phenomenon could influence arrestin interactions in the cytoplasmic leaflet when CDase hydrolyzes ceramide on the extracellular leaflet.

While acid and neutral CDases have been identified in mammals, *Drosophila* encodes only one neutral Cdase, and no acid CDase homolog has been identified. While a null mutation of neutral CDase is lethal in *Drosophila*, mice whose corresponding homolog is disrupted do not show obvious abnormalities (Kono et al., 2006). Rather, they have a normal lifespan with no major alterations in ceramide levels in their tissues, but they are deficient in the intestinal degradation of ceramide. Knockout mice for acid CDase, however, die as embryos, and the heterozygotes show progressive lipid storage disease (Li et al., 2002). It is tempting to speculate that neutral CDase may also play the role of acid CDase in *Drosophila*.

In conclusion, we found that CDase functions in a cell-nonautonomous manner to maintain photoreceptor homeostasis. Furthermore, these results show the functional coupling of the sphingolipid biosynthetic pathway with *Drosophila* photoreceptor survival and function.

EXPERIMENTAL PROCEDURES

Fly Stocks and Husbandry

Drosophila stocks were cultured on standard corn meal agar and maintained at 25°C unless otherwise mentioned. The *Df (3R) tll-g*/TM6B (stock # 2599) was obtained for Bloomington Stock Center, Indiana; fat body driver (C564) flies were obtained from Neal Silverman, UMass Medical School, Worcester; mushroom body driver flies were obtained from Scott Waddell, UMass Medical School, Worcester; GMR p35 flies were from Bruce Hay, California Institute of Technology, Pasadena; *arr2*^{3} and *ninaE*^{I17}flies were from Charles Zuker, University of California, San Diego. *crb*^{11A22} neoFRT82B flies were from Ulrich Tepass, University of Toronto, Ontario and Kwang-wook Choi, Baylor College of Medicine, Houston. Eye-specific cdase^{-} mosaic clones were generated by crossing ey-FLP; ; neoFRT82Bw^{+}cl3R3/TM6B to w;neoFRT82B*cdase*^{1}/TM6B. For experiments in the light, flies were maintained in a 12 hr light and 12 hr dark cycle, whereas, for experiments in the dark, flies were maintained in complete darkness. For transgenic rescue of *Drosophila* null mutants, a 10 kb genomic fragment of *Drosophila* CDase including 4.4 kb of promoter region and 1 kb downstream of the gene was amplified by PCR, cloned into pUAST vector, clones identified, sequenced, and transgenic flies were generated. One of these mapped to the second chromosome and was used in transgenic rescue experiments. The transgenic expression of CDase protein was confirmed by western analysis in the null mutant background.

Genetic Screen and Isolation of *cdase* Mutants

The genetic scheme and method are described in the Supplemental Data. After isolation of *cdase* lethal mutants, they were backcrossed three times to *w1118* flies and selected by transgenic rescue experiments to outcross all incidental and irrelevant mutations.

Immunohistochemistry

Electron Microscopy

For all electron microscopic examination, unless otherwise specified, 5-day-old flies grown at 25°C were decapitated under anesthesia and their heads dissected, fixed, and processed as described before (Acharya et al., 2003).

Immunostaining

All antibodies used for staining and conditions for staining discs and photoreceptors are described in the Supplemental Data.

Ceramide Quantitation

For lipid extraction, flies frozen at −20°C were homogenized in methanol:-chloroform (2:1) in the presence of an internal standard, sonicated, and the mix was incubated overnight at 37°C. The extract was saponified to hydrolyze phospholipids and purified over a SepPak C18 reverse phase column. The lipid samples were subjected to ESI/MS/MS analysis as described previously (Acharya et al., 2003; Thomas et al., 1999). ESI mass spectral analyses of ceramides were performed utilizing a Waters Quatro II Triple Quadruple Mass Spectrometer equipped with a z-spray electrospray ion source. Mass-lynx (version 3.5) software was used for data analysis. We found that homogenization of flies in organic solvent and the subsequent extraction of lipids overnight at 37°C resulted in better yields of sphingolipids than extraction in aqueous solvent, which was used in our earlier methods. This also resulted in higher signal for ceramide in the mass spectral analysis.

Arrestin Binding and Release Assays

Arrestin binding assays were performed as described previously (Alloway et al., 2000). For Arr binding assays, five heads were dissected from less than 2-day-old dark-reared adults and added to a buffer containing 150 mM KCl, 20 mM Tris (pH 7.5), 5 mM DTT, and Protease Inhibitor Cocktail Tablet (Roche). Blue and orange light were generated using a 150 W High Intensity Illuminator, Nikon NI 150 that were filtered through blue and orange Photo Visual Color filters (Meade Instruments Corp., USA). For binding assays, fly heads were exposed to 2 min of blue light, homogenized, centrifuged at 13,000 g for 5 min and pellet and supernatant fractions separated under dim red light. Samples were then incubated at 37°C for 10 min and subjected to SDS/PAGE and western analysis. Arrestin release assays were performed in the same manner except that isolated fly heads were exposed to 2 min of blue light followed by 4 min of orange light, prior to homogenization and centrifugation.

ERG Recordings

ERGs were performed using two glass microelectrodes filled with 1 M NaCl. The recording electrode was placed on the surface of the fly eye, while the ground electrode was inserted into the thorax of the fly. White light stimulation from a xenon arc lamp (Lambda LS 175W, Sutter Instruments, Novato, CA) was passed through a 400–700 nm bandpass filter and focused on the specimen using a fiber optic light guide. Signals were amplified by a differential DAM 50 amplifier (World Precision Instruments, Sarasota, FL) and sampled at 1 kHz using a Digidata 1322A digitizer (Axon Instruments/Molecular Devices, Sunnyvale, CA). To remove high-frequency noise, data were filtered using a boxcar smoothing filter (Clampfit, Axon Instruments/Molecular Devices, Sunnyvale, CA). All experiments were conducted at room temperature.

Cell Culture

Culturing of *Drosophila* S2 cells and generation of V5 CDase stable cell lines are described in the Supplemental Data.

Internalization Experiments with V5 CDase

One of the stable cell lines expressing tagged CDase was induced with $CuCl_2$ for 72 hr, and extracellular media was collected. V5-tagged CDase was enriched by 50% ammonium sulfate precipitation of the media followed by extensive dialysis. For internalization experiments, 3-day-old S2 cells were washed and incubated with the dialyzed protein fraction for different time periods. The cells were then plated on poly lysine-coated coverslips, fixed, blocked, and stained with different primary antibodies. V5 antibody was used at 1:300, Rab11 at 1:750, and Alexa secondary antibodies were used at 1:2000.

C12-NBD Ceramide Labeling of S2 Cells

A 10 μM C12-NBD-ceramide (Avanti Polar Lipids, Inc., Alabaster, AL) labeling solution was prepared in cold Schneider media containing 2% serum, and 1×10^6 S2 cells were incubated for 1 hr on ice in the labeling solution. Cells were then centrifuged at 1200 rpm, washed to remove excess label, and incubated with or without V5 CDase at 25°C for 2 hr. Lipids were extracted, applied on preadsorbent TLC plate (Whatman, Clifton, NJ), and developed with chloroform:methanol:ammonia (90:20:0.5). The plates were dried and scanned with Fujifilm FLA-5000 Phosphor-imager in fluorescence mode. Quantitation was performed with Fujifilm ImageGauge software version 4.2.

RNAi Treatment of S2 Cells

dsRNA treatments were carried out as described earlier (Clemens et al., 2000). The details for making dsRNA to Cdase and RNAi treatment are described in the Supplemental Data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/57/1/69/DC1/.

ACKNOWLEDGMENTS

We thank Drs. Robert Cohen, Marcos Gonzalez-Gaitin, Sean Munro, John Sisson, and Charles Zuker for their kind gifts of antibodies. We also thank the Bloomington Stock Center, Drs. Kwang-wook Choi, Bruce Hay, Ulrich Tepass, Scott Waddell, and Charles Zuker for fly stocks. We would also like to thank Dr. Neal Silverman for helpful discussions and Dr. Andrea Pereira for advice on dissecting fat bodies. We thank Jason de la Cruz for excellent technical assistance. We thank Drs. Ira Daar, Michael Green, and Shyam Sharan for their comments on the manuscript. This work is supported by NIH grant (RO1EY16469) and a Child Health Research grant from Charles H. Hood Foundation to U.A.; by the intramural Research Program of NCI, NIH, HHS (J.K.A.); by NIH grant (RO1EY013751) to S.T.

Received: April 30, 2007
Revised: September 17, 2007
Accepted: October 30, 2007
Published: January 9, 2008

REFERENCES

Acharya, U., and Acharya, J.K. (2005). Enzymes of sphingolipid metabolism in *Drosophila* melanogaster. Cell. Mol. Life Sci. *62*, 128–142.

Acharya, U., Patel, S., Koundakjian, E., Nagashima, K., Han, X., and Acharya, J.K. (2003). Modulating sphingolipid biosynthetic pathway rescues photoreceptor degeneration. Science *299*, 1740–1743.

Acharya, U., Mowen, M.B., Nagashima, K., and Acharya, J.K. (2004). Ceramidase expression facilitates membrane turnover and endocytosis of rhodopsin in photoreceptors. Proc. Natl. Acad. Sci. USA *101*, 1922–1926.

Acharya, U., Edwards, M.B., Jorquera, R.A., Silva, H., Nagashima, K., Labarca, P., and Acharya, J.K. (2006). *Drosophila* melanogaster Scramblases modulate synaptic transmission. J. Cell Biol. *173*, 69–82.

Adachi-Yamada, T., Gotoh, T., Sugimura, I., Nishida, Y., Onuki, T., and Date, H. (1999). De novo synthesis of sphingolipids is required for cell survival by down-regulating c-Jun N-terminal kinase in Drosophila imaginal discs. Mol. Cell. Biol. *19*, 7276–7286.

Alloway, P.G., Howard, L., and Dolph, P.J. (2000). The formation of stable rhodopsin-arrestin complexes induces apoptosis and photoreceptor cell degeneration. Neuron *28*, 129–138.

Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S.S., Stefansson, S., Liau, G., and Hla, T. (2002). Extracellular export of sphingosine kinase-1

enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. J. Biol. Chem. *277*, 6667–6675.

Chen, C.S., Martin, O.C., and Pagano, R.E. (1995). Ceramide as a modulator of endocytosis. J. Biol. Chem. *270*, 13291–13297.

Chuang, J.Z., Vega, C., Jun, W., and Sung, C.H. (2004). Structural and functional impairment of endocytic pathways by retinitis pigmentosa mutant rhodopsin-arrestin complexes. J. Clin. Invest. *114*, 131–140.

Claing, A., Laporte, S.A., Caron, M.G., and Lefkowitz, R.J. (2002). Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. Prog. Neurobiol. *66*, 61–79.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA. *97*, 6499–6503.

Colley, N.J., Baker, E.K., Stamnes, M.A., and Zuker, C.S. (1991). The cyclophilin homolog ninaA is required in the secretory pathway. Cell *67*, 255–263.

Colley, N.J., Cassill, J.A., Baker, E.K., and Zuker, C.S. (1995). Defective intracellular transport is the molecular basis of rhodopsin-dependent dominant retinal degeneration. Proc. Natl. Acad. Sci. USA *92*, 3070–3074.

D'Hondt, K., Heese-Peck, A., and Riezman, H. (2000). Protein and lipid requirements for endocytosis. Annu. Rev. Genet. *34*, 255–295.

Dickson, R.C. (1998). Sphingolipid functions in Saccharomyces cerevisiae: comparison to mammals. Annu. Rev. Biochem. *67*, 27–48.

Dollar, G., Struckhoff, E., Michaud, J., and Cohen, R.S. (2002). Rab11 polarization of the *Drosophila* oocyte: a novel link between membrane trafficking, microtubule organization, and oskar mRNA localization and translation. Development *129*, 517–526.

Futerman, A.H., and Hannun, Y.A. (2004). The complex life of simple sphingolipids. EMBO Rep. *5*, 777–782.

Futerman, A.H., and Riezman, H. (2005). The ins and outs of sphingolipid synthesis. Trends Cell Biol. *15*, 312–318.

Hannun, Y.A., and Luberto, C. (2000). Ceramide in the eukaryotic stress reponse. Trends Cell Biol. *10*, 73–80.

Hannun, Y.A., and Obeid, L.M. (2002). The ceramide–centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. J. Biol. Chem. *277*, 25847–25850.

Hardie, R.C., and Raghu, P. (2001). Visual transduction in *Drosophila*. Nature *413*, 186–193.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. Development *120*, 2121–2129.

Hla, T. (2004). Physiological and pathological actions of sphingosine 1-phosphate. Semin. Cell Dev. Biol. *15*, 513–520.

Holthuis, J.C., Pomorski, T., Raggers, R.J., Sprong, H., and Van Meer, G. (2001). The organizing potential of sphingolipids in intracellular membrane transport. Physiol. Rev. *81*, 1689–1723.

Hrdlicka, L., Gibson, M., Kiger, A., Micchelli, C., Schober, M., Schock, F., and Perrimon, N. (2002). Analysis of twenty-four Gal4 lines in *Drosophila melanogaster*. Genesis *34*, 51–57.

Izaddoost, S., Nam, S.C., Bhat, M.A., Bellen, H.J., and Choi, K.W. (2002). Drosophila Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. Nature *416*, 178–183.

Kiselev, A., Socolich, M., Vinos, J., Hardy, R.W., Zuker, C.S., and Ranganathan, R. (2000). A molecular pathway for light-dependent photoreceptor apoptosis in *Drosophila*. Neuron *28*, 139–152.

Kolesnick, R., and Fuks, Z. (2003). Radiation and ceramide-induced apoptosis. Oncogene *22*, 5897–5906.

Kono, M., Dreier, J.L., Ellis, J.M., Allende, M.L., Kalkofen, D.N., Sanders, K.M., Bielawski, J., Bielawska, A., Hannun, Y.A., and Proia, R.L. (2006). Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. J. Biol. Chem. *281*, 7324–7331.

Kumar, J.P., and Ready, D.F. (1995). Rhodopsin plays an essential structural role in *Drosophila* photoreceptor development. Development *121*, 4359–4370.

Kurada, P., and O'Tousa, J.E. (1995). Retinal degeneration caused by dominant rhodopsin mutations in *Drosophila*. Neuron *14*, 571–579.

Le Stunff, H., Milstien, S., and Spiegel, S. (2004). Generation and metabolism of bioactive sphingosine 1-phosphate. J. Cell. Biochem. *92*, 882–899.

Li, C.M., Park, J.H., Simonaro, C.M., He, X., Gordon, R.E., Friedman, A.H., Ehleiter, D., Paris, F., Manova, K., Hepbildikler, S., et al. (2002). Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage diseases in heterozygotes. Genomics *79*, 218–224.

Marks, D.L., Singh, R.D., Choudhury, A., Wheatley, C.L., and Pagano, R.E. (2005). Use of fluorescent sphingolipid analogs to study lipid transport along the endocytic pathway. Methods *36*, 186–195.

Montell, C. (1999). Visual transduction in *Drosophila*. Annu. Rev. Cell Dev. Biol. *15*, 231–268.

Mitsutake, S., and Igarashi, Y. (2007). Transbilayer movement of ceramide in the plasma membrane of live cells. Biochem. Biophys. Res. Commun. *359*, 622–627.

Newsome, T.P., Asling, B., and Dickson, B.J. (2000). Analysis of photoreceptor axon guidance in eye-specific mosaics. Development *127*, 851–860.

Orem, N.R., Xia, L., and Dolph, P.J. (2006). An essential role for endocytosis of rhodopsin through interaction of visual arrestin with the AP-2 adaptor. J. Cell Sci. *119*, 3141–3148.

Pellikka, M., Tanentzapf, G., Pinto, M., Smith, C., McGlade, C.J., Ready, D.F., and Tepass, U. (2002). Crumbs, the Drosophila homologue of human CRB1/RP12 is essential for photoreceptor morphogenesis. Nature *416*, 143–149.

Rohrbough, J., Rushton, E., Palanker, L., Woodruff, E., Matthies, H.J., Acharya, U., Acharya, J.K., and Broadie, K. (2004). Ceramidase regulates synaptic vesicle exocytosis and trafficking. J. Neurosci. *24*, 7789–7803.

Satoh, A.K., and Ready, D.F. (2005). Arrestin 1 mediates light-dependent rhodopsin endocytosis and cell survival. Curr. Biol. *15*, 1722–1733.

Schwaerzel, M., Heisenberg, M., and Zars, T. (2002). Extinction antagonizes olfactory memory at the subcellular level. Neuron *35*, 951–960.

Sisson, J.C., Field, C., Ventura, R., Royou, A., and Sullivan, W. (2000). Lava lamp, a novel peripheral golgi protein, is required for Drosophila melanogaster cellularization. J. Cell Biol. *151*, 905–918.

Spiegel, S., and Milstien, S. (2003). Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem. Soc. Trans. *31*, 1216–1219.

Stark, W.S., Sapp, R., and Schilly, D. (1988). Rhabdomere turnover and rhodopsin cycle: maintenance of retinula cells in *Drosophila* melanogaster. J. Neurocytol. *17*, 499–509.

Sugita, M., Dulaney, J.T., and Moser, H.W. (1972). Ceramidase deficiency in Farber's disease (lipogranulomatosis). Science *178*, 1100–1102.

Tani, M., Iida, H., and Ito, M. (2003). O-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein. J. Biol. Chem. *278*, 10523–10530.

Thomas, R.L., Jr., Matsko, C.M., Lotze, M.T., and Amoscato, A.A. (1999). Mass spectrometric identification of increased C16 ceramide levels during apoptosis. J. Biol. Chem. *274*, 30580–30588.

Webel, R., Menon, I., O'Tousa, J.E., and Colley, N.J. (2000). Role of asparagine-linked oligosaccharides in rhodopsin maturation and association with its molecular chaperone NinaA. J. Biol. Chem. *275*, 24752–24759.

Wucherpfennig, T., Wilsch-Brauninger, M., and Gonzalez-Gaitan, M. (2003). Role of *Drosophila* Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J. Cell Biol. *161*, 609–624.

Yoshimura, Y., Okino, N., Tani, M., and Ito, M. (2002). Molecular cloning and characterization of a secretory neutral ceramidase of *Drosophila* melanogaster. J. Biochem. (Tokyo) *132*, 229–236.

Zuker, C.S. (1996). The biology of vision in *Drosophila*. Proc. Natl. Acad. Sci. USA *93*, 571–576.